Survival and stimulation of neurite outgrowth in a serum-free culture of spiral ganglion neurons from adult mice

Mauricio Vieira [a,b] (vieira@uiuc.edu), Barbara L. Christensen [a,c] (blchris@uiuc.edu), Bruce C. Wheeler [a,b,d] (bwheeler@uiuc.edu), Albert S. Feng [a,c] (afeng1@uiuc.edu), and Richard Kollmar [a,c,*] (rkollmar@uiuc.edu)

[a] *Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign, 405 North Mathews Avenue, MC-251, Urbana, IL, USA*

[b] *Department of Bioengineering, University of Illinois at Urbana-Champaign, 1304 West Springfield Avenue, MC-278, Urbana, IL 61801, USA*

[c] *Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, 407 South Goodwin Avenue, MC-114, Urbana, IL 61801, USA*

[d] *Department of Electrical and Computer Engineering, University of Illinois at Urbana-Champaign, 1406 West Green Street, MC-702, Urbana, IL 61801, USA*

[*] *Corresponding Author:*

407 South Goodwin Avenue, MC-114

Urbana, IL 61801

USA

Telephone: (217) 333-9861

Facsimile: (217) 333-1133

E-mail: rkollmar@uiuc.edu

Abstract

We have developed a reliable protocol for the serum-free dissociation and culture of spiral ganglion neurons from adult mice, an important animal model for patients with post-lingual hearing loss. Pilot experiments indicated that the viability of spiral ganglion cells *in vitro* depended critically on the use of Hibernate medium with B27 supplement. With an optimized protocol, we obtained $2 \cdot 10^3$ neurons immediately after dissociation, or about one-fifth of those present in the intact spiral ganglion. After four days in culture, 4% of the seeded neurons survived without any exogenous growth factors other than insulin. This yield was highly reproducible in five independent experiments and enabled us to measure systematically the numbers and lengths of the regenerating neurites. Furthermore, the survival rate compared well to the few published protocols for culturing adult spiral ganglion neurons from other species. Enhanced survival and neurite outgrowth upon the addition of brain-derived neurotrophic factor and leukemia inhibitory factor demonstrated that both are potent stimulants for damaged spiral ganglion neurons in adults. This responsiveness to exogenous growth factors suggested that our culture protocol will facilitate the screening of molecular compounds as potential treatments for sensorineural hearing loss.

Keywords

Adult

Cell Culture Techniques

Mice

Neurites

Neurons

Spiral Ganglion

1. Introduction

The damage caused to spiral ganglion neurons in patients with sensorineural hearing loss may be halted or reversed by using cochlear implants or gene therapy to supply peptide growth factors (Holley, 2002). However, our understanding of the signaling events that underlie the survival of spiral ganglion neurons and the regeneration of their neurites is incomplete and requires further studies to guide technology development (Bianchi and Raz, 2004; Gillespie and Shepherd, 2005; Webber and Raz, 2006). The primary culture of dissociated spiral ganglion neurons from adult mice would be a useful addition to the experimental armamentarium for such studies. First, initial studies are often easier to analyze and require fewer animals when conducted *in vitro* rather than *in vivo*. Second, the anatomy, physiology, and pathology of hearing are well-documented for mice and similar to those of humans (Nadol, 1988; Ohlemiller, 2006; Willott, 2001); unlike other mammalian animal models, mice offer a full range of molecular-genetic tools, mutant and transgenic lines, and inbred strains with varying degrees of hearing loss (Zheng et al., 1999). Finally, adult animals are the most appropriate model for adult patients with postlingual deafness, who comprise the largest group with sensorineural hearing loss (Collins, 1997; Gates and Mills, 2005).

No protocol has been published for the primary culture of spiral ganglion neurons from adult mice. The pioneering work on culturing dissociated spiral ganglion neurons was carried out more than a decade ago with embryonic rats and chicken (Lefebvre et al., 1990a; Yamaguchi and Ohmori, 1990). Various protocols have since been established for culturing spiral ganglion neurons from a range of species and ages, such as embryonic mice (Rabejac et al., 1994; Vazquez et al., 1994); neonatal mice (Kita et al., 2005; Lin et al., 1998; Mo and Davis, 1997; Whitlon et al., 2006), rats (Dazert et al., 1998; Hegarty et al., 1997; Lefebvre et al., 1990b; Malgrange et al., 1996; Marzella et al., 1997; Ripoll and Rebillard, 1997; Rome et al., 1999; Zheng et al., 1995), and gerbils (Lin, 1997); and adult rats (Lefebvre et al., 1991), guinea pigs (Anderson et al., 2006; Rask-Andersen et al., 2005), and humans (Rask-Andersen et al., 2005). Sensory epithelia have also been cultured from the cochlea of adult guinea pigs (Zhao, 2001); most experiments with spiral

ganglion neurons, however, have been conducted with neonatal samples, possibly because adult neurons are in general more difficult to culture (Banker and Goslin, 1998).

The Hibernate and Neurobasal media and the B27 supplement were developed specifically for the serum-free isolation and culture of neurons, in part by optimizing the osmolarity and by including antioxidants (Brewer et al., 1993; Brewer, 1997). In contrast, almost all protocols cited above use physiological saline for the isolation of spiral ganglion neurons and generic chemically-defined media, such as Dulbecco modified Eagle medium, together with the traditional N1 or N2 supplements (Bottenstein and Sato, 1985) for their culture. Only a single study has described culturing spiral ganglion neurons in Neurobasal medium with B27 supplement (Anderson et al., 2006), but the quantitation of survival and neurite outgrowth was not the focus of that work. Furthermore, most of the cited protocols require fetal bovine serum during the dissociation or even in the culture media. This may improve neuronal survival, but may also confound the effects of specific growth factors added as experimental treatments with the effects of growth factors present in serum.

We developed a protocol for the completely serum-free dissociation and culture of spiral ganglion neurons from adult mice in Hibernate and Neurobasal media with B27 supplement. Our protocol reproducibly yielded sufficient numbers of neurons for systematic measurements of cell numbers and neurite lengths. Enhanced survival and neurite outgrowth upon the addition of neuronal growth factors demonstrated that this protocol will be useful for studying the regeneration of adult spiral ganglion neurons.

2. Materials and methods

2.1. Dissociating spiral ganglion neurons

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign. Adult mice, strain CBA/CaJ (The Jackson Laboratory, Bar Harbor, ME), were decapitated between postnatal days 30 and 72, and both temporal bones were resected. Subsequent steps were performed in a sterile hood at room temperature, unless stated otherwise. The temporal bones were washed three times in 1 ml of Hibernate A (Brain Bits, Springfield, IL) supplemented with 2% (v/v) B27 (Invitrogen, Carlsbad, CA) and kept immersed in the same medium. The cochlea was separated from the vestibular apparatus, the bony capsule opened, and the modiolus excised. After the organ of Corti had been peeled away, the modiolus was split longitudinally to expose the spiral ganglion and transferred into 1 ml Hibernate A plus B27 in a collection tube at 0°C. Twelve cochleas were collected for each experiment, with an average dissection time of 20 min per animal. After all animals had been processed, the dissected modioli were washed together once in 3 ml Hibernate A alone by centrifugation at 200 × *g* for 1 min and resuspended in the same volume of Hibernate A containing 5 units/ml dispase (Calbiochem, San Diego, CA) and 0.5 mg/ml collagenase I (Sigma, Saint Louis, MO). The tissue was incubated for 30 min at 33°C with gentle agitation after every 5 min. The proteases were quenched by adding bovine serum albumin (BSA; Sigma) to 1 mg/ml, and the modioli were washed again by centrifugation in 3 ml Hibernate A plus B27. The tissue was resuspended in 1 ml Hibernate A plus B27 and dissociated by aspirating and expelling 30 times through the 1.4-mm orifice of a 1000-µl polypropylene pipet tip (Fisher Scientific, Hampton, NH). Tissue clumps were allowed to sediment for 1-2 minutes, and the supernatant was collected. The dissociation and sedimentation steps were repeated two more times. To determine the initial yield, the cells in the combined supernatants were counted with a hemocytometer under differential-interference-contrast illumination (Fig. 1A). Dead cells were revealed by trypan blue staining, and large cells with a diameter of about

20-25 μm were scored as spiral ganglion neurons, whether or not they sported neurite stubs (Ripoll and Rebillard, 1997). The dissociated cells were pelleted by centrifugation at 200 × *g* for 3 min and resuspended at $1.33 \cdot 10^4$ neurons/ml in culture medium consisting of Neurobasal A (Invitrogen), 0.5 mM glutamine, 25 μM glutamate, 2% (v/v) B27, 100 U/ml penicillin, and 10 μg/ml streptomycin (both from Sigma).

2.2. Culturing dissociated spiral ganglion neurons

The bottoms of 80-mm^2 wells in tissue-culture-treated polystyrene plates (Corning-Costar, Acton, MA) were coated first in 300 μl of 0.1 mg/ml poly-D-lysine (Sigma) for 16 hours at room temperature, rinsed twice with phosphate-buffered saline, then coated in 300 μl of 10 μg/ml laminin (Sigma) in Neurobasal A for 6 hours at 33°C in a 5% CO_2 atmosphere, and again rinsed twice. About one hour before seeding (before washing the dissected modioli with Hibernate A alone, see above), each well received 150 μl culture medium containing no additional growth factors (treatment 'None'), 20 or 40 ng/ml recombinant human brain-derived neurotrophic factor (BDNF; Promega, Madison, WI), 100 or 200 ng/ml leukemia inhibitory factor (LIF; Sigma), 20 or 40 ng/ml recombinant human neurotrophin-3 (NTF3 or NT3; Promega), or a combination of the three factors at these concentrations (treatment 'All Three'). At the end of the dissociation, $2 \cdot 10^3$ spiral ganglion neurons in 150 μl culture medium were added to each well, reducing the final BDNF, LIF, or NTF3 concentrations to one-half of those above. The cultures were kept in a 5% CO_2 atmosphere, first at 33°C for 16 hours and then at 37°C for 80 hours. For immunofluorescence detection, cultures were seeded at the same density onto chambered glass slides (Lab-Tek; Nalge Nunc, Rochester, NY).

2.3. Evaluating cultures of spiral ganglion neurons

Cultured spiral ganglion neurons were visualized by immunocytochemistry (Harlow and Lane, 1999) with a monoclonal mouse antibody (TuJ1; Covance, Denver, PA) against the neuronal marker β-III tubulin (Hallworth and Luduena, 2000). The fixative was 4% (w/v) formaldehyde in a buffer containing 60 mM PIPES, 50 mM

HEPES, 20 mM EGTA, 4 mM $MgCl_2$ (pH 7.2); the permeabilization buffer was 0.3% (v/v) triton X-100 in phosphate-buffered saline; the primary antibody and the secondary horseradish-peroxidase-conjugated goat anti-mouse antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) were diluted 1:500 and 1:1000, respectively, in saline containing 1.5% (w/v) BSA; the color reaction with diaminobenzidine was enhanced with Ni^{2+} (Vector Laboratories, Burlingame, CA); and the stained samples were stored at -20°C in a polyvinyl-alcohol-based mounting medium (Fluoromount; Southern Biotech, Birmingham, AL) until further analysis. For immunofluorescence detection, a polyclonal rabbit antiserum against bovine heavy-polypeptide neurofilament (neurofilament 200; Sigma) was included in some experiments, the secondary antibodies were fluorescein-isothiocyanate-conjugated donkey anti-rabbit and tetramethylrhodamine-5-isothiocyanate-conjugated donkey anti-mouse (Jackson) diluted 1:200, and nuclei were labeled with 2 μg/ml nuclear yellow (Invitrogen). In control samples without primary antibodies, no staining was observed by the secondary antibodies alone at equivalent durations of the color reaction or camera exposures. Furthermore, the fluorescent anti-rabbit antibody did not cross-react with the primary mouse antibody and *vice versa,* and emission from each fluorophore was detected only in its respective fluorescence channel.

To discriminate between healthy and dying neurons, fragmented DNA was visualized with a kit for *in situ* terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL; Roche Diagnostics, Indianapolis, IN). In control reactions, no signal was observed after TUNEL reactions without terminal deoxynucleotidyl transferase, whereas all nuclei were labeled after a partial digest with 30 units/ml DNase I.

To count the neurons and the number of neurites per neuron, all brown-to-black-stained β-III tubulin-positive cells in a well with large (about 20–25 μm diameter) and roundish somata were scored (compare Fig. 1B). To measure neurite lengths, only those neurons were scored whose processes did not overlap or touch the side of the well; this may have biased our results towards cells with shorter neurites. The neurons were photographed individually under differential-interference-contrast illumination with a

digital camera connected to an inverted microscope. The images were analyzed by measuring the longest branch of each neurite with the NeuronJ plugin for the ImageJ software (Meijering et al., 2004). Five independent experiments with duplicate wells for each treatment were evaluated by using two-way analysis of variance and Bonferroni-corrected pairwise comparisons. Multicolor fluorescence images were acquired with epifluorescence optics; each field of view was also photographed under phase contrast illumination.

3. Results

3.1. Dissociation and culture of spiral ganglion neurons from adult mice

To dissociate and culture spiral ganglion neurons from adult mice, we adapted a protocol that had been developed for hippocampal neurons from adult rats (Brewer et al., 1993; Brewer, 1997). This protocol is similar to those developed earlier for spiral ganglion neurons, but incorporates several innovations to maximize neuronal survival (see INTRODUCTION). A series of pilot experiments (data not shown) suggested that the viability of our cultures was greatly improved by dissecting and dissociating in Hibernate A medium supplemented with B27 rather than in physiological saline and by pairing collagenase with the metalloprotease dispase instead of the sulfhydryl protease papain during the dissociation. We also found that the proteases could be quenched with purified BSA instead of complete serum, thus eliminating exposure of the spiral ganglion neurons to any exogenous growth factors other than the insulin that is contributed by B27 and essential for the survival of neurons in culture. Further improvements were achieved by using Neurobasal with B27 and 25 μM glutamate (Balazs, 2006; Konur and Ghosh, 2005) as the culture medium and by incubating the cultures initially at 33°C before transfer to 37°C (Whitlon et al., 2006). On the fourth day of culture without external growth factors, the neurons appeared healthy, with large, roundish somata and neither pyknotic nuclei (Fig. 1C, left section) nor DNA strand breaks (Fig. 1C, right section). In cultures maintained for one week, the number of spiral ganglion neurons decreased by about 20% between days four and seven. The details of our final protocol are given under MATERIALS AND METHODS.

To quantify the performance and reproducibility of our protocol, we then measured the number of neurons, the number of neurites, and the length of the neurites in five independent experiments. Immediately after dissociation, the yield was $(1.99 \pm 0.10) \cdot 10^3$ spiral ganglion neurons per cochlea (mean ± standard error; $n = 4$), of which 94% ± 5% were alive. About equal numbers of these neurons bore no or only short processes; at this stage, we never observed cells with processes longer than a few

micrometers. After four days of culture in the absence of exogenous growth factors, each well contained a number of neurons that corresponded to about 4% of those seeded initially (Table 1, treatment 'None') and a similar number of non-neuronal cells. About one third of the neurons sported one or more neurites (Fig. 2A, 'None'). The mean length of the longest neurite on each neuron was about 50 μm (Fig. 2B, 'None'), and the maximum length was 1.3 mm (see Fig. 1B for an example). Labeling with the monoclonal antibody against β-III tubulin was specific: It was limited to cells with the large (about 20-25 μm diameter), roundish soma characteristic of spiral ganglion neurons (Figs. 1B & D, 'Tubb3') and coincided with labeling by an antibody against heavy-polypeptide neurofilament, another well-established neuronal marker (compare 'Tubb3' and 'Nfh' in Fig. 1E). Even in overdeveloped samples with weakly positive non-neuronal cells, bipolar non-myelinating Schwann cells could easily be distinguished by their smaller (less than 10 μm diameter) and spindle-shaped soma (Fig. 1B, right enlargement). We concluded from these results that our protocol allowed some of the dissociated spiral ganglion neurons from adult mice to survive and to regenerate neurites in the absence of exogenous growth factors.

3.2. Responsiveness of cultured spiral ganglion neurons to exogenous growth factors

To ascertain that the signaling pathways of the spiral ganglion neurons were not saturated by endogenous growth factors secreted from glial or other types of cells in our cultures and that survival and neurite outgrowth could be stimulated further by adding exogenous growth factors, we added BDNF, LIF, or NTF3 to the medium, each of which is known to protect cultured spiral ganglion neurons and stimulate neurite outgrowth (Gillespie and Shepherd, 2005). An initial dose-response analysis suggested optimal concentration ranges of 10-20 ng/ml for BDNF and NTF3, and 100-200 ng/ml for LIF (data not shown) that were consistent with previous reports (Lefebvre et al., 1994; Marzella et al., 1997; Mou et al., 1997; Whitlon et al., 2006; Zheng et al., 1995). Each of the three growth factors alone significantly improved the survival of spiral ganglion neurons over four days in culture (Table 1). BDNF or LIF alone, but not NTF3, significantly increased both

the number of neurites per neuron (Fig. 2A) and the mean length of the longest neurite per neuron (Fig. 2B). Within the narrow range applied, growth-factor concentration affected only neurite length. The effects on neurite outgrowth were additive—both the neurite number and the longest length were greatest in medium that contained all three growth factors together. These results demonstrated that some of the cultured adult spiral ganglion neurons remained responsive to exogenous growth factors.

4. Discussion

In this study, we developed a completely serum-free protocol for the dissociation and culture of spiral ganglion neurons from adult mice. The degree of survival and the extent of neurite outgrowth were highly reproducible. Furthermore, the cultured spiral ganglion neurons remained responsive to exogenous growth factors.

The initial yield of spiral ganglion cells after dissociation and their survival in culture depended critically on the use of both Hibernate medium and B27 supplement during the isolation. Processing a sufficient number of mice took us several hours, but cochlear afferents suffer from oxidative stress already after 20 min of ischemia (Pujol et al., 1992). The combination of Hibernate and B27 has been formulated to extend the viability of neurons in storage to several days (Brewer and Price, 1996). Moreover, the antioxidant components of B27–catalase, glutathione, superoxide dismutase, and vitamin E (Brewer et al., 1993)–have been shown to be essential for the long-term survival of cultured cortical neurons (Perry et al., 2004). The presence of these antioxidants may thus account for the improved yield over physiological saline as the isolation medium.

Our yields were very reproducible and compared well to the few published protocols for culturing adult spiral ganglia without any exogenous growth factors other than insulin: We counted $2 \cdot 10^3$ neurons per spiral ganglion immediately after dissociation, or about 20% of the $0.8\text{-}1.2 \cdot 10^4$ neurons found in the intact spiral ganglion of adult mice (Ehret, 1979; Whitlon et al., 2006); 4% of the seeded neurons, or about 80 per spiral ganglion, survived after four days in culture. By comparison, the yield after dissociation was 10% for adult rats, and 1% to 6% of the seeded neurons survived (Lefebvre et al., 1991); 20-30 neurons per spiral ganglion survived for adult guinea pigs (Anderson et al., 2006). On an absolute scale, all these yields are low, as may be expected for adult neurons (Banker and Goslin, 1998).

Our culture experiments with exogenous BDNF, LIF, and NTF3 provide new insights into the responsiveness of adult spiral ganglion neurons to neuronal growth factors. LIF has been known to enhance survival and neurite outgrowth in cultures

derived from neonatal mice and rats (Gillespie et al., 2001; Marzella et al., 1997; Whitlon et al., 2006), but ours is the first report of a stimulatory effect on cultures derived from adult animals. BDNF and NTF3 have been shown previously to promote survival and neurite outgrowth upon ototoxic damage *in vivo* (Gillespie and Shepherd, 2005); *in vitro,* however, with spiral ganglion neurons from adult rats, they have been found to promote survival only (Lefebvre et al., 1994). In contrast, we observed in our cultures that BDNF stimulated both survival and neurite outgrowth; this discrepancy may have been due to the differences in age, species, and culture conditions. Our results thus demonstrate that LIF and BDNF are both potent growth factors that could be used to treat damaged spiral ganglion neurons in adults.

Two caveats apply to our work as well as to previous studies with dissociated spiral ganglia. First, the inevitable presence of other cell types introduces an uncontrolled variable. The reported ratios of non-neuronal, mostly glial cells to spiral ganglion neurons range from 1:1 (this study) to 20:1 (Rask-Andersen et al., 2005). Both glial-conditioned media and purified glial-derived neurotrophic factor (GDNF) promote the survival and sprouting of spiral ganglion neurons (Gillespie and Shepherd, 2005). Effects of exogenous growth factors on survival and neurite outgrowth may thus be mediated or augmented by the secretion of endogenous factors from non-neuronal cells.

The second caveat is that some or all of the neurons observed at the end of the culture period could be newly-differentiated stem or progenitor cells instead of surviving and regenerating mature neurons. Earlier culture studies with adult spiral ganglia did not have to consider this possibility because neural stem cells for the longest time were thought to exist in just a few locations elsewhere in the adult nervous system. Only recently have neural stem cells been isolated from spiral ganglia of adult humans and guinea pigs (Rask-Andersen et al., 2005). On the other hand, neural stem cells have not been detected in spiral ganglia of mice older than 6-8 weeks (Lopez et al., 2004; Oshima et al., 2007). Nonetheless, neural stem cells and regenerating mature neurons are equally important as therapeutic targets in the adult spiral ganglion; the presence of stem cells in a culture would not diminish its value as an experimental model.

In spite of the uncertainties about contributions from non-neuronal cells and neural stem cells, establishing a reliable culture protocol for spiral ganglion neurons from adult mice has been an important step towards screening growth factors and other molecular compounds as potential treatments for sensorineural hearing loss.

Note Added in Proof

While this manuscript was under review, a protocol was published for the culture of spiral ganglion neurons from adult mice that uses fetal bovine serum (Wei, D., Jin, Z., Jarlebark, L., Scarfone, E., Ulfendahl, M. 2006. Survival, synaptogenesis, and regeneration of adult mouse spiral ganglion neurons in vitro. J. Neurobiol. 67, 108–122).

Acknowledgements

We thank Holly Fairfield, Milan Mulye, Luke Pro, Samit Shah, and Ben-Paul Umunna for assistance; Dr. Donna Whitlon for discussions at the outset of this project; and Samit Shah and the members of the Kollmar lab for comments on the manuscript. This work was supported by funds from the Beckman Institute for Advanced Science and Technology, the Campus Research Board, and the Mary Jane Neer Fund at the University of Illinois at Urbana-Champaign.

References

Anderson, M., Bostrom, M., Pfaller, K., Glueckert, R., Schrott-Fischer, A., Gerdin, B., Rask-Andersen, H., 2006. Structure and locomotion of adult *in vitro* regenerated spiral ganglion growth cones - A study using video microscopy and SEM. Hear. Res. 215, 97-107.

Balazs, R., 2006. Trophic effect of glutamate. Curr. Top. Med. Chem. 6, 961-968.

Banker, G., Goslin, K., 1998. Culturing Nerve Cells, 2nd ed., MIT Press, Cambridge, MA.

Bianchi, L.M., Raz, Y., 2004. Methods for providing therapeutic agents to treat damaged spiral ganglion neurons. Curr. Drug Targets CNS Neurol. Disord. 3, 195-199.

Bottenstein, J.E., Sato, G., 1985. Cell Culture in the Neurosciences, Plenum Press, New York, NY.

Brewer, G.J., Torricelli, J.R., Evege, E.K., Price, P.J., 1993. Optimized survival of hippocampal neurons in B27-supplemented neurobasal™, a new serum-free medium combination. J. Neurosci. Res. 35, 567-576.

Brewer, G.J., Price, P.J., 1996. Viable cultured neurons in ambient carbon dioxide and hibernation storage for a month. Neuroreport 7, 1509-1512.

Brewer, G.J., 1997. Isolation and culture of adult rat hippocampal neurons. J. Neurosci. Methods 71, 143-155.

Collins, J.G., 1997. Prevalence of selected chronic conditions: United States, 1990-1992. Vital and health statistics, 1-89.

Dazert, S., Kim, D., Luo, L., Aletsee, C., Garfunkel, S., Maciag, T., Baird, A., Ryan, A.F., 1998. Focal delivery of fibroblast growth factor-1 by transfected cells induces spiral ganglion neurite targeting *in vitro*. J. Cell. Physiol. 177, 123-129.

Ehret, G., 1979. Quantitative analysis of nerve fibre densities in the cochlea of the house mouse *(Mus musculus)*. J. Comp. Neurol. 183, 73-88.

Gates, G.A., Mills, J.H., 2005. Presbycusis. Lancet 366, 1111-1120.

Gillespie, L.N., Clark, G.M., Bartlett, P.F., Marzella, P.L., 2001. LIF is more potent than BDNF in promoting neurite outgrowth of mammalian auditory neurons *in vitro*. Neuroreport 12, 275-279.

Gillespie, L.N., Shepherd, R.K., 2005. Clinical application of neurotrophic factors: the potential for primary auditory neuron protection. Eur. J. Neurosci. 22, 2123-2133.

Hallworth, R., Ludueña, R.F., 2000. Differential expression of β tubulin isotypes in the adult gerbil cochlea. Hear. Res. 148, 161-172.

Harlow, E., Lane, D., 1999. Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hegarty, J.L., Kay, A.R., Green, S.H., 1997. Trophic support of cultured spiral ganglion neurons by depolarization exceeds and is additive with that by neurotrophins or cAMP and requires elevation of $[Ca^{2+}]_i$ within a set range. J. Neurosci. 17, 1959-1970.

Holley, M.C., 2002. Application of new biological approaches to stimulate sensory repair and protection. Br. Med. Bull. 63, 157-169.

Kita, T., Nakagawa, T., Kim, T.S., Iwai, K., Takebayashi, S., Akaike, A., Ito, J., 2005. Serofendic acid promotes survival of auditory hair cells and neurons of mice. Neuroreport 16, 689-692.

Konur, S., Ghosh, A., 2005. Calcium signaling and the control of dendritic development. Neuron 5, 401-405.

Lefebvre, P.P., Leprince, P., Weber, T., Rigo, J.M., Delree, P., Moonen, G., 1990a. Neuronotrophic effect of developing otic vesicle on cochleo-vestibular neurons: evidence for nerve growth factor involvement. Brain Res. 507, 254-260.

Lefebvre, P.P., Weber, T., Rigo, J.M., Delree, P., Leprince, P., Moonen, G., 1990b. Potassium-induced release of an endogenous toxic activity for outer hair cells and auditory neurons in the cochlea: a new pathophysiological mechanism in Meniere's disease? Hear. Res. 47, 83-93.

Lefebvre, P.P., Van de Water, T.R., Weber, T., Rogister, B., Moonen, G., 1991. Growth factor interactions in cultures of dissociated adult acoustic ganglia: neuronotrophic effects. Brain Res. 567, 306-312.

Lefebvre, P.P., Malgrange, B., Staecker, H., Moghadass, M., Van de Water, T.R., Moonen, G., 1994. Neurotrophins affect survival and neuritogenesis by adult injured auditory neurons *in vitro*. Neuroreport 5, 865-868.

Lin, X., 1997. Action potentials and underlying voltage-dependent currents studied in cultured spiral ganglion neurons of the postnatal gerbil. Hear. Res. 108, 157-179.

Lin, X., Chen, S., Tee, D., 1998. Effects of quinine on the excitability and voltage-dependent currents of isolated spiral ganglion neurons in culture. J. Neurophysiol. 79, 2503-2512.

Lopez, I.A., Zhao, P.M., Yamaguchi, M., Vellis, J.d., Espinosa-Jeffrey, A., 2004. Stem/progenitor cells in the postnatal inner ear of the GFP-nestin transgenic mouse. Int. J.Dev. Neurosci. 22, 205-213.

Malgrange, B., Lefebvre, P., Van de Water, T.R., Staecker, H., Moonen, G., 1996. Effects of neurotrophins on early auditory neurones in cell culture. Neuroreport 7, 913-917.

Marzella, P.L., Clark, G.M., Shepherd, R.K., Bartlett, P.F., Kilpatrick, T.J., 1997. LIF potentiates the NT-3-mediated survival of spiral ganglia neurones *in vitro*. Neuroreport 8, 1641-1644.

Meijering, E., Jacob, M., Sarria, J.C., Steiner, P., Hirling, H., Unser, M., 2004. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. Cytometry A 58, 167-176.

Mo, Z.L., Davis, R.L., 1997. Endogenous firing patterns of murine spiral ganglion neurons. J. Neurophysiol. 77, 1294-1305.

Mou, K., Hunsberger, C.L., Cleary, J.M., Davis, R.L., 1997. Synergistic effects of BDNF and NT-3 on postnatal spiral ganglion neurons. J. Comp. Neurol. 386, 529-539.

Nadol, J.B., Jr., 1988. Comparative anatomy of the cochlea and auditory nerve in mammals. Hear. Res. 34, 253-266.

Ohlemiller, K.K., 2006. Contributions of mouse models to understanding of age- and noise-related hearing loss. Brain Res. 1091, 89-102.

Oshima, K., Grimm, C.M., Corrales, C.E., Senn, P., Martinez Monedero, R., Geleoc, G.S., Edge, A., Holt, J.R., Heller, S., 2007. Differential distribution of stem cells in the auditory and vestibular organs of the inner ear. J. Assoc. Res. Otolaryngol. 8:18-31.

Perry, S.W., Norman, J.P., Litzburg, A., Gelbard, H.A., 2004. Antioxidants are required during the early critical period, but not later, for neuronal survival. J. Neurosci. Res. 78, 485-492.

Pujol, R., Puel, J.L., Eybalin, M., 1992. Implication of non-NMDA and NMDA receptors in cochlear ischemia. Neuroreport 3, 299-302.

Rabejac, D., Raymond, J., Dechesne, C.J., 1994. Characterization of different neuron populations in mouse statoacoustic ganglion cultures. Brain Res. 652, 249-256.

Rask-Andersen, H., Bostrom, M., Gerdin, B., Kinnefors, A., Nyberg, G., Engstrand, T., Miller, J.M., Lindholm, D., 2005. Regeneration of human auditory nerve. *In vitro*/*in video* demonstration of neural progenitor cells in adult human and guinea pig spiral ganglion. Hear. Res. 203, 180.

Ripoll, C., Rebillard, G., 1997. A simple technique to efficiently dissociate primary auditory neurons from 5 day-old rat cochleas. J. Neurosci. Meth. 73, 123-128.

Rome, C., Luo, D., Dulon, D., 1999. Muscarinic receptor-mediated calcium signaling in spiral ganglion neurons of the mammalian cochlea. Brain Res. 846, 196-203.

Vazquez, E., Van de Water, T.R., Del Valle, M., Vega, J.A., Staecker, H., Giraldez, F., Represa, J., 1994. Pattern of trkB protein-like immunoreactivity *in vivo* and the *in vitro* effects of brain-derived neurotrophic factor (BDNF) on developing cochlear and vestibular neurons. Anat. Embryol. (Berl.) 189, 157-167.

Webber, A., Raz, Y., 2006. Axon guidance cues in auditory development. Anat. Rec. A Discov. Mol. Cell. Evol. Biol. 288, 390-396.

Whitlon, D.S., Ketels, K.V., Coulson, M.T., Williams, T., Grover, M., Edpao, W., Richter, C.P., 2006. Survival and morphology of auditory neurons in dissociated cultures of newborn mouse spiral ganglion. Neuroscience 138, 653-662.

Willott, J.F., 2001. Handbook of Mouse Auditory Research: From Behavior to Molecular Biology, CRC Press, Boca Raton, FL.

Yamaguchi, K., Ohmori, H., 1990. Voltage-gated and chemically gated ionic channels in the cultured cochlear ganglion neurone of the chick. J. Physiol. 420, 185-206.

Zhao, H.-B., 2001. Long-term natural culture of cochlear sensory epithelia of guinea pigs. Neurosci. Lett. 315, 73-76.

Zheng, J.L., Stewart, R.R., Gao, W.Q., 1995. Neurotrophin-4/5 enhances survival of cultured spiral ganglion neurons and protects them from cisplatin neurotoxicity. J. Neurosci. 15, 5079-5087.

Zheng, Q.Y., Johnson, K.R., Erway, L.C., 1999. Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. Hear. Res. 130, 94-107.

Figure Captions

Fig. 1. Spiral ganglion neurons from adult mice after dissociation and after four days in culture without exogenous growth factors. *(A)* Trunks of neurons *(arrowheads)* among debris immediately after dissociation and labeling with trypan blue. *DIC,* differential interference contrast illumination; *, dead neuron. *(B)* Example of a cultured neuron, with the neuronal marker β-III tubulin *(Tubb3)* stained dark brown in a color reaction. The framed areas have been enlarged threefold: *middle section,* the neuron's large, roundish, dark soma next to a faintly-stained fibroblast or astrocyte; *right section,* a weakly-stained cell with a small, spindle-shaped soma *(arrow),* most likely a bipolar non-myelinating Schwann cell. *(C-E)* Cultured neurons and non-neuronal cells distinguished by concurrent fluorescent labeling of β-III tubulin *(red)* and nuclei *(blue).* In *C,* fragmented DNA had also been labeled to detect apoptosis *(TUNEL, green),* but only small, non-neuronal nuclei were positive *(*).* In *E,* the localization of β-III tubulin was compared to that of heavy-polypeptide neurofilament *(Nfh, green);* the fluorescence of both neuronal markers coincided in a neuron *(arrowhead),* but was not observed in non-myelinating Schwann cells *(arrows).* Note the higher density of non-neuronal cells on the glass substrate used for the cultures shown in *C-E. Phase,* phase contrast illumination. The scale bar in *A* corresponds to 30 μm in *A & B* and to 10 μm in the enlargements in *B*; the scale bar in *C* corresponds to 15 μm in *C* and to 30 μm in *D & E.*

Fig. 2. Neurite outgrowth from spiral ganglion neurons in the absence or presence of exogenous growth factors. *(A)* Number of neurites per cell after four days in culture; mean of ten wells containing a total of 778–1223 neurons per growth-factor treatment. *(B)* Length of the longest neurite per cell at low (10 ng/ml BDNF and LIF, 100 ng/ml NTF3) or high (20 ng/ml BDNF and LIF, 200 ng/ml NTF3) growth-factor concentrations; mean of 55–156 cells per treatment whose neurites did not overlap or touch the side of the well. The key to the growth-factor treatments in *A* also applies to panel *B;* error bars, standard error of the mean; *, significantly different from treatment 'None' ($P \leq 0.03$).

Table

Table 1

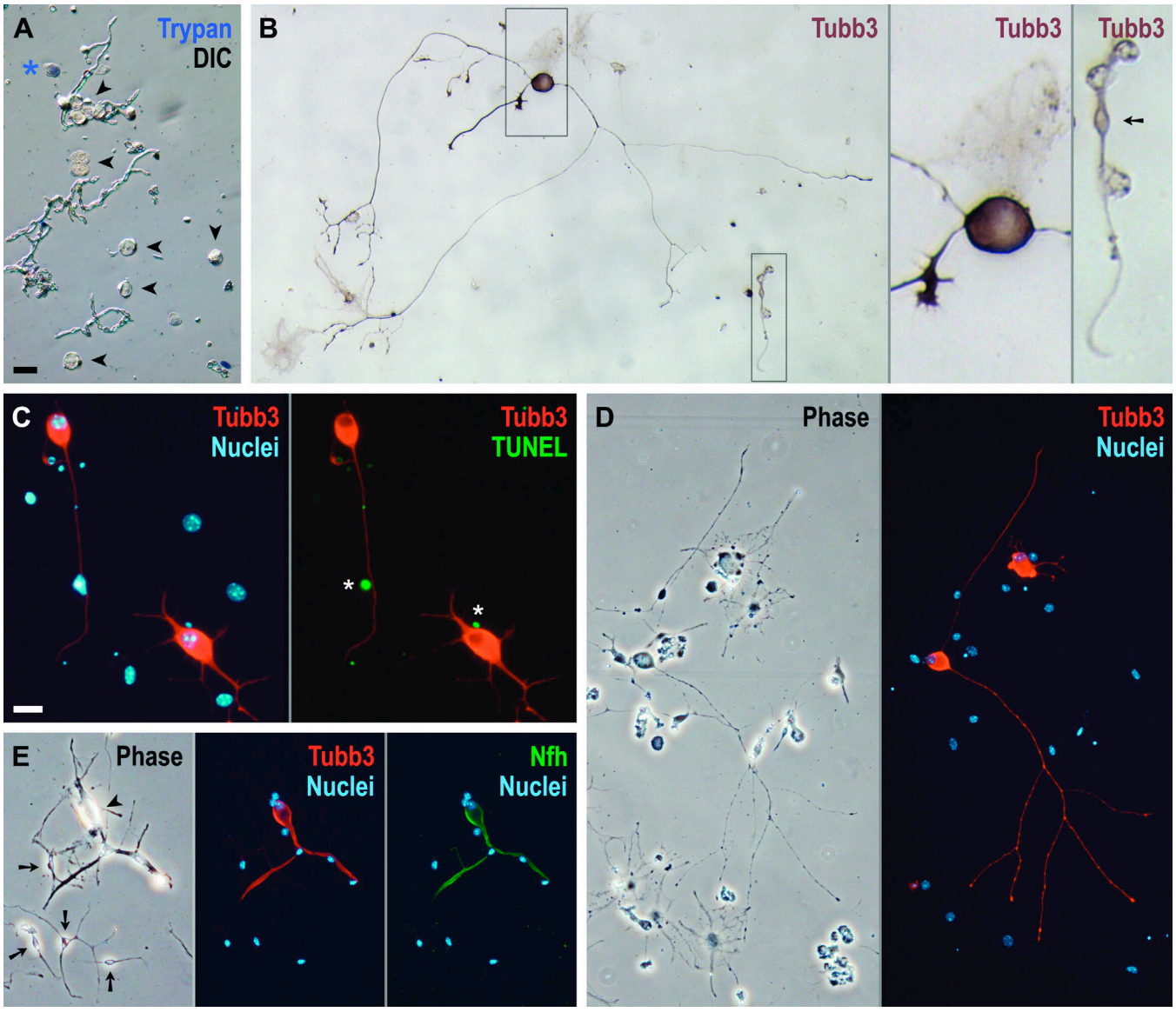
Survival of spiral ganglion neurons from adult mice

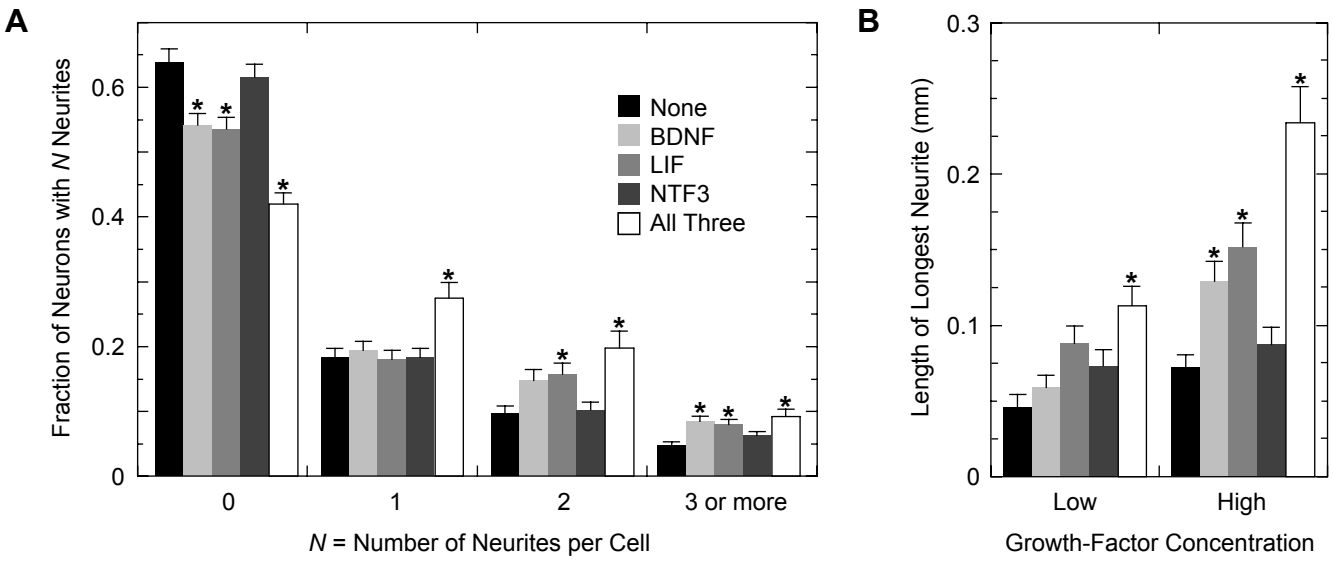
Growth-Factor Treatment	Neurons per 80-mm^2 Well[†]
None	76 ± 4 (10)
BDNF	118 ± 6 (10)[**]
LIF	110 ± 5 (10)[**]
NTF3	95 ± 5 (10)[*]
All Three	108 ± 6 (8)[**]

[†] Mean ± standard error *(n)* after culturing a seed of $2 \cdot 10^3$ neurons for four days in serum-free medium.

[**] Significantly different from 'None' ($P \leq 10^{-4}$).

[*] Significantly different from 'None' ($P < 0.02$).


A
Trypan
DIC
B
Tubb3
Tubb3
Tubb3
C
Tubb3
Nuclei
Tubb3
TUNEL
D
Phase
Tubb3
Nuclei
E
Phase
Tubb3
Nuclei
Nfh
Nuclei


A
Fraction of Neurons with N Neurites
0
0.2
0.4
0.6
None
BDNF
LIF
NTF3
All Three
0
1
2
3 or more
N = Number of Neurites per Cell
B
Length of Longest Neurite (mm)
0
0.1
0.2
0.3
Low
High
Growth-Factor Concentration